PROTOCOL NAME: Protein aggregation capture, clean-up and on-bead digestion followed by fractionation using MagReSyn® SAX

PROTOCOL ID: SAX-FRACTIONATION

DATE LAST MODIFIED: 24 January 2020

INTRODUCTION:

The protocol describes the use of protein aggregation capture (PAC, Baath *et al*) to capture and clean-up 200 µg of lysate with using Strong Anion Exchange (SAX) magnetic beads. Proteins are extracted and solubilized in Urea, followed by efficient on-bead Trypsin digestion. Peptides are subsequently eluted with stepwise pH-based fractionation to partition samples for LC-MS analysis. This fractionation allows the potential for increased proteome coverage. The method is scalable as long as the protein:bead ratio is kept at 1:10. A minimum bead quantity of 100 µg, 5µl (suitable for 10 µg of protein) is required for good particle retention on a magnetic separator.

IMPORTANT NOTES:

- *The preferred method of protein preparation for this protocol is cell lysis by sonication in urea (8M Urea, 50 mm Tris pH 8). Samples should be clarified by centrifugation and treated with Benzonase (refer manufacturers recommendations for your sample). Urea will require dilution to below 5M to ensure Benzonase activity.*
- *This protocol is suitable for the samples extracted in urea or GdHCl, but is **NOT** compatible for clean-up of samples extracted in detergents such as **SDS** due to possible accumulation on the SAX magnetic beads and elution at acidic pH.*
- *If the sample is not suitable for PAC, e.g. sample such as urine is of low concentration that does not allow for efficient precipitation, alkaline pH can be used as an alternate to capture and concentrate the proteins.*
- *The current methods are suitable for manual preparation, or may be fully automated on magnetic bead handling systems such as KingFisher™ or similar, protocols are available on request.*
- *The protocol is sufficient for the clean-up of 200 µg of protein, but can be linearly scaled for higher and lower protein quantities but ensure the bead ratio remains constant at 1:10.*
- *Please contact info@resynbio.com if you have any queries relating to this protocol or to inquire about sample preparation techniques compatible with your samples.*

MATERIALS: All reagents and chemicals should be of analytical grade or better, and preferably MS grade.

- MagReSyn® SAX – Catalogue Number MR-SAX002
- Eppendorf LoBind microcentrifuge tubes, 0.5, 1.5 and 2 ml.
- Magnetic Separator or Magnetic bead handling station (e.g. KingFisher™)
- Pipettes
- Ammonium bicarbonate (NH_4Bicarb)
- Ammonium acetate (NH_4Acetate)
- Ammonium formate (NH_4Formate)
- Trifluoracetic acid (TFA)
- Iodoacetamide (IAA)
- Dithiothreitol (DTT)
- Peptide quantification kit (e.g. Pierce™ Quantitative Colorimetric Peptide Assay)
- MS grade water

REAGENT PREPARATION: **NOTE**: *Buffers can be stored at 4°C for up to 2 weeks.*

- ***DTT Stock***: 1M DTT (prepare fresh)
- ***IAA Stock***: 1M IAA (prepare fresh, light sensitive)
- ***Equilibration Buffer***: 70% Acetonitrile
- ***Wash Buffer***: 95% Acetonitrile
- ***Trypsin Digestion Buffer***: 50 mM NH_4Bicarb
- ***Elution Buffers:*** Prepare elution buffers of varying pH's by adjusting buffers with TFA according to the table on the right.

Name	pH	Volume	Buffer	TFA (µl)
Elution Buffer 1	7	5	50mM NH_4Bicarb	7
Elution Buffer 2	6	5	50mM NH_4Bicarb	17
Elution Buffer 3	5	5	50mM NH_4Acetate	5.5
Elution Buffer 4	4	5	50mM NH_4Acetate	15
Elution Buffer 5	3	5	50mM NH_4Formate	19.5
Elution Buffer 6	2	5	50mM NH_4Formate	30
Elution Buffer 7	1	1	1% TFA	NA

METHOD:

PROTEIN LYSATE PREPARATION:

1. Pellet mammalian cells at 200-500 g for 10 min or bacteria at 3000-5000 g for 10 min
2. Re-suspend cells in lysis buffer (8M Urea, 50 mM Tris pH 8; 200 µl fine for 0.5 million cell pellet)
3. Sonicate cells on ice, 9 pulses (10 sec per pulse with 10 sec intermission between pulses; 50% power setting)
4. Dilute samples so that urea concentration is at 5M
5. Add 25 units of benzonase per 0.5million cells and $MgCl_2$ to a final of 2 mM

   Note: urea concentration needs to be ≥ 6M for benzonase to retain activity
6. Incubate at 35 °C for 30min
7. Centrifuge at 10,000 g for 10 min to clear cell debris
8. Repeat steps 5-7 if slight buffy layer still evident
9. Collect supernatant and determine concentration using BCA assay

   Note: urea concentration needs to be ≥ 3M to avoid interference with BCA assay
10. Prepare 200 µg aliquots and store at -80 °C

    On day of analysis:
11. Thaw lysates on ice
12. Reduce proteins using DTT to a final of 10 mM for 30 min at 35 °C
13. Alkylate proteins using IAA to a final of 40 mM for 30 min in the dark

MICROPARTICLE EQUILIBRATION:

1. Re-suspend MagReSyn® SAX thoroughly by vortex mixing or inversion to ensure a homogenous suspension. **NOTE**: *When multiple samples are being prepared, ensure that you maintain a homogeneous suspension by mixing regularly, for example by inversion or pipetting the micro particle mixture up/down before transferring the required volume.*
2. Transfer 100 µl MagReSyn® SAX (2 mg) microparticles to a 2 ml Protein Lo-Bind tube.
3. Place the tube on a magnetic separator and allow 5-10 sec for the microparticles to clear.
4. Remove the shipping solution by aspiration with a pipette and discard.
5. Wash the microparticles by re-suspending in 200 µl of ***Equilibration Buffer*** (70% Acetonitrile) with agitation (e.g. gentle vortex mixing) for 1 min.
6. Place the tube on the magnetic separator and allow the microparticles to clear.
7. Remove the equilibration solution by aspiration with a pipette and discard.
8. Repeat steps **5 – 7** for a further 2 equilibrations.

PROTEIN CAPTURE AND ON-BEAD DIGESTION:

9. Add protein sample from **13** (200 µg) to beads from **8** (2 mg) above. **NOTE***: a minimum volume of 25 µl is recommended to use for manual processing*
10. Add Acetontrile to achieve a final concentration of 70%, incubate for 10 min at room temperature without agitation to allow for aggregation.
11. Place the tube on the magnetic separator and allow the microparticles to clear. Remove and discard the unbound fraction by aspiration with a pipette. **NOTE**: *you can retain the unbound fraction and quantify for potentially unbound proteins using a suitable protein quantification technique or SDS PAGE.*
12. Retain the bead-protein pellet on the magnetic separator and add 500 µl of ***Wash Solution*** (95% Acetonitrile).
13. Remove the supernatant after 30 seconds without disturbing the pellet and discard.
14. Repeat steps **12** and **13**. **NOTE**: *in addition to Acetonitrile, a range of solvents can be utilized including EtOH, MeOH, or isopropanol to remove possible contaminants, but ensure that solvent concentration is sufficient to ensure protein aggregation.*
15. Perform on-bead digestion by adding in 100 µl of 50 mM Ammonium Bicarbonate pH 8.0 containing 20 µg sequencing grade Trypsin (1:10 enzyme:protein ratio), mix to form a uniform suspension and digest for 1 hr at 47°C. Ensure sufficient mixing to keep the particles in solution during digestion to ensure good particle liquid interaction.

    NOTE: *Less trypsin ratio can be used if longer digest times are used.*
16. Place the tube on a magnetic separator and allow 5-10 sec for the microparticles to clear.
17. Remove peptide solution and place in a 0.5 ml Eppendorf LoBind tube. This constitutes **Fraction 1**, unbound peptides suitable for LCMS analysis.
18. Sequentially elute peptides by adding **Elution Buffer 1 to 7** (decreasing pH) for and label **Fraction 2 to Fraction 8**, for LCMS analysis. Agitate for 5 min for each stepwise elution with recovery by magnetic separator.

NOTES:

- *Samples can be vacuum or freeze-dried to reduce the volume of the sample from frozen at low temperature, and resuspended in 40 ul of 0.2% Formic Acid with 2% ACN.*
- *To estimate peptide recovery for LC MS analysis you can use a peptide quantification kit such as Pierce™ Quantitative Colorimetric Peptide Assay.*
- *Sample can be desalted with C18 SPE or on-line with a C18 trap cartridge in a typical pre-concentration LCMS set-up.*

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